Developing a Crystal Violet Assay to Quantify Biofilm Production Capabilities of *Staphylococcus aureus*

Honors Research Thesis

Presented in Partial Fulfillment of the Requirements for Graduation with
Honors Research Distinction

Antoinette Metzler

Department of Animal Sciences
The Ohio State University

2016

Project Advisors
Dr. Armando Hoet, DMV, PhD
Diplomate ACVPM
Associate Professor
Coordinator, Veterinary Public Health Program
Department of Veterinary Preventive Medicine
A100Q Sisson Hall
1920 Coffey Rd
Columbus, OH 43210
Hoet.1@osu.edu

ABSTRACT

Staphylococcus aureus is an opportunistic pathogen responsible for significant morbidity and mortality in clinical settings in both humans and animals. This emerging pathogen is among the top three nosocomial pathogens in human and veterinary hospitals due to its ability to survive in these environments for long periods. This survival is likely the result of *S. aureus*'s production of biofilm: a protective matrix of bacterially secreted proteins that allow colonies to attach to environmental surfaces. Preventing and controlling this pathogen, specifically within small animal veterinary hospitals, becomes critical for two reasons. One, the presence of this pathogen increases the risk of animals developing a hospital-acquired infection. Two, this pathogen poses an occupational risk to veterinary hospital staff. Therefore, it is important to know the biofilm production potential and characteristics of *S. aureus* isolates in these animal facilities. This information can be used to more effectively prevent or control a uniquely natured biofilm-producing *S. aureus*. To quantify biofilm production potential of *S. aureus* isolates the Crystal Violet (CV) assay is commonly used. This assay is preferred due to its simplicity, reliability, and quick throughput. With this assay isolates can be categorized as high, moderate, or non-biofilm producers. To adapt this assay, we used two high producer bacterial control strains NE95 and NE1241, a moderate producer wild type JE2 (WT), and a low producer NE1193 to validate the CV assay. Assay optimization included changes in the quantity of overday culture growth, overnight incubation time, aspiration quantities, washing technique, and microplate reader absorbance wavelength. Through assay optimization a sensitive and specific CV assay was developed for the quantification and characterization of *S. aureus* biofilm production.

INTRODUCTION

Staphylococcus aureus is an opportunistic pathogen responsible for significant health problems in humans[1] and animals[2]. In addition to morbidity and mortality, it causes increased treatment fees, prolonged hospitalization, frustration, and grief. Rising reports of *S. aureus* infections make this pathogen now among the top nosocomial pathogens in human hospitals.[3] This rise in reports is particularly concerning due to its bidirectional transmissibility between humans and animals in household, community, and healthcare settings.[4] Further heightening this concern are reports of *S. aureus*'s prevalence in veterinary hospitals. In one study of a veterinary hospital, *S. aureus* was reported to contaminate one in every five, or 18-20%, of contact surfaces.[3] Van Balen et al.[3] also reported that *S. aureus* can survive on inanimate objects and contact surfaces of veterinary origin for up to seven months. In a veterinary environment, *S. aureus* isolates have also been shown to circulate between multiple surfaces and areas within a

hospital for up to nine months. This prolonged environmental contamination exposes both animal patients and hospital staff, further increasing both staff risk of occupational related colonization and patient risk of hospital-acquired infection.[3] Therefore, *S. aureus* is becoming a serious animal and public health concern.

In human medicine, *S. aureus*'s survival in the environment is at least in part due to its production of biofilm. In general, biofilms are surface-associated microbial communities encapsulated by a protective matrix of bacterially secreted carbohydrates, proteins, and DNA. In vivo, microbial cells within a biofilm are protected from the host immune system and acquire an enhanced resistance to antibiotics.[5] In fact, it is reported that once a biofilm reaches its climax community, normal antimicrobial treatment concentrations become ineffective at eradication.[6] Biofilms can increase *S. aureus's* antibiotic resistance up to 1,000 fold.[7] In clinical environments, complex microbial communities within biofilm have enhanced cell-to-cell communications, allowing for rapid adaption to changing environments. For these reasons, in human healthcare settings, biofilms are therefore considered to be an important virulence factor of *S. aureus*[8] and can complicate the control and eradication of *S. aureus*.[7]

To begin to understand how to control these biofilm-producing pathogens in hospital settings, it is first necessary to develop a complete understanding of biofilm formation. Briefly, in order for a microorganism to form biofilm a bacterially secreted matrix and adherable surface must be present. Development of a biofilm involves two phases, attachment and maturation, which lead to a complex switch in growth mode, from planktonic (floating) to sessile (attached).[7] Initially, attachment to a surface is promoted by specific bacterial adhesions called microbial surface components recognizing adhesive matrix molecules (MSCRAMMS). Once attached, the maturation phase follows and bacteria accumulate as they adhere to each other and begin to

secrete the matrix of carbohydrates, proteins, and DNA. Genetically, *S. aureus* forms a biofilm when the accessory gene regulator (*agr*) locus that encodes a quorum-sensing system is down regulated to allow for attachment. Additionally, the regulatory locus staphylococcal accessory regulator (*sarA*) is expressed due to its importance in the control of the intracellular adhesion (*ica*) operon[9], which is vital for biofilm development. Products of the *ica* operon are responsible for the biosynthesis of polysaccharide intercellular adhesin (PIA), the main molecule used in intercellular adhesion in staphylococci.[10]

It is known that human hospital strains of *S. aureus* can produce a biofilm that enables their prolonged environmental presence and increased microbial resistance. Therefore, research has been conducted on ways to fight and eradicate biofilm-producing pathogens, by taking into account the unique features of biofilm when developing disinfectant protocols.[11] These same advancements to cleaning procedures have not occurred in veterinary medicine because currently, little research exists on the potential of veterinary hospital strains of *S. aureus* to produce biofilms. Recent increases in veterinary surface contamination as well as the long-term survival of *S. aureus* in veterinary environments necessitate that this research be conducted.[3] It is hypothesized that, because of epidemiological similarities between *S. aureus* strains isolated from a veterinary hospitals and human hospitals, veterinary strains will similarly be capable of producing biofilm. The results of this research will aid in the protection of both veterinary hospital staff and patients by ensuring that the control of zoonotic transmission takes into account all virulent characteristic of this potentially deadly pathogen.

In order to quantify the biofilm production capabilities of an isolate, the Crystal Violet (CV) assay is often preferred due to its simplicity, reliability, and quick throughput. This method allows for the *in vitro* cultivation and quantification of bacterial biofilms.[12] The CV

nonspecifically stains all biomass, both living and dead, as well as the matrix composed of extracellular polymeric substances.[13] This stain makes the assay useful to assess the overall biofilm response of an isolate.[12] Through this method, an isolate can be classified as high, moderate, or non-biofilm producer. The objective of this study was to optimize the Crystal Violet phenotypic biofilm screening technique for *S. aureus*. In doing so, high, moderate, and non-biofilm producing controls were validated for future assays for which clinical isolates can be statistically compared to controls and characterized as high, moderate, or non-producers. The information obtained about these clinical isolates of veterinary origin will help to better understand the potential role of biofilm production in the ecology and epidemiology of *S. aureus* in veterinary hospitals. This information can then be used to revise and enhance cleaning and disinfecting protocols to better ensure the safety of patients and hospital staff.

PROCEDURES AND METHODS

Control Strains

Two well characterized high biofilm producing *S. aureus* strains (Sa^+), NE95 and NE1241, one well characterized moderate biofilm producing *S. aureus* strain, wild type JE2 (WT), and one well characterized non-biofilm producing *S. aureus* strain (Sa^-), NE1193, were obtained and used to optimize CV protocol.

High Producers

NE95 (*agr*-) is a high biofilm producing isolate. It has been found that the presence of this quorum-sensing accessory gene regulator (*agr*) locus is responsible for cell-to-cell communication. The presence of this locus inhibits the attachment and development of biofilm. By mutating this locus, NE95 is known to form more robust biofilms compared to wild type strains and thus, have improved biofilm development capabilities.[14]

NE1241 (*nucA*-) lacks a *nucA* gene that is known to control the expression of the enzyme Thermonuclease (*nuc*). It has been shown that suppression or removal of *nuc* enhances biofilm formation, promoting the high producing phenotype.[15] There is therefore an inverse correlation between *nuc* activity and biofilm formation.[16]

Moderate Producer

JE2 (WT) is a wild-type strain of *S. aureus* known to produce a moderate amount of biofilm under proper conditions.[10, 15]

Low Producer

NE1193 (*sarA*-) lacks the accessory regulatory A (*sarA*) locus, which is involved in the regulation of extracellular and cell wall proteins in *S. aureus*.[17] For this reason mutation of *sarA* results in a reduced biofilm formation capacity of *S. aureus*.[18]

Crystal Violet Assay

The developed CV assay was adapted from the Microbial Infection and Immunity Microbiology Center for Microbial Interface Biology, The Ohio State University. Briefly, the isolates were first inoculated on Trypticase Soy Agar (TSA), a growth medium, and incubated overnight at 37°C. Then, 1 colony was added to 5mL of Trypticase Soy Broth (TSB) and grown overnight at 37 °C in shaker at 200 rpm. 50 uL of overnight culture was then added to 5 mL of TSB and an overday culture was grown at 37°C for 2-3 hours in a shaker at 200 rpm. Using a spectrophotometer blanked with TSB, the overday incubation was stopped when the OD_{600} was between 0.5 and 0.7. Each well of a 96-well tissue culture plate was then inoculated with 200 uL of overday culture at ~0.5 OD_{600}; 6 wells/plate/isolate for 3 repetitions. The plate was then grown statically at 37°C overnight. After, 180 uL of each well of the 96-well plate was aspirated and the plate was washed in

large beaker of water for 5 rigorous passes. It was then blotted on paper towels 2-5 times. Next, 200 uL of 0.1% aqueous CV was added to each well and the plate was left to stand on the bench for 30 minutes. 180 uL of each well of the 96-well plate was aspirated again and then the plate was washed in large beaker of water for 5 rigorous passes. It was then blotted on paper towels 2-5 times. To elute the bound CV, 200 uL of 95% ethanol was added to each well and the plate was left to stand on the bench for 30 minutes. Lastly, the lid was removed and the plate is read with a microplate reader at 540 nm. This CV assay was optimized for equipment in the Diagnostic and Research Laboratory on Infectious Diseases (DRLID), The Ohio State University.

Data Analysis

To analyze the results of the plate readings, a one-way ANOVA test is run with a Bonferroni post-hoc analysis. The one-way ANOVA test is used to determine if there are significant differences between the means of plated isolates. The Bonferroni post- hoc analysis allows for comparison between data groups within the plate. The CV assay control strains are preforming optimally when there is significant difference between the high, moderate, and non-producers.

RESULTS AND DISCUSSION

To ensure the CV technique performed sensitively, specifically, and reliably optimization of the quantity of overday culture growth, overnight incubation time, aspiration quantities, washing technique, and microplate reader absorbance wavelength were required.

Overday Culture

The original CV assay required the use of a test tube direct read spectrophotometer, which read the turbidity of the overday culture directly from the incubated test tube. In the

current study, a spectrophotometer requiring 2 mL cuvette subsamples for each reading was used. It was found that on average, two subsample readings were taken before the desired 0.5 OD is reached, increased the odds of running out of sample for plating. Therefore, the protocol was revised as follows: 90 uL of overnight culture is added to 9 mL of TSB. This revision preserves the overnight:TSB ratio of the original protocol, 50 uL of overnight to 5 mL of overday or 0.01%, and provides enough volume for subsampling and plating. However, as seen in Table 1, this new volume of overday culture volume took an average of 4.919 hours, rather than the expected 2 to 3 hours, to reach an OD_{600} of ~0.5. We suspected that the increased volume (5 mL to 9 mL) in the 10 mL test tube did not provide the culture with optimal oxygen for an exponential growth response. To resolve this issue, the protocol was further modified 125 mL Erlenmeyer flasks were used in place of 10 mL test tubes. To these, 400 uL of overnight culture is added 40mL of TSB. This modification resulted in the return of the expected overday growth time of 2.722 hours on average as seen in Table 2.

Table 1: Average overday incubation time
90 uL (culture):9 mL (TSB)

Run	Date	To Reach OD_{600} (hrs)
1	10.12	5.000
2	10.21	4.925
3	10.29	4.833
		4.919

Table 2: Average overday incubation time
400 uL (culture): 400 mL (TSB)

Run	Date	To Reach OD_{600} (hrs)
1	3.2	2.833
2	3.7	3.000
3	4.12	2.333
		2.722

Overnight Incubation Time

The overnight incubation time of the 96-well plate was increased from 18 to 24 hours due to a recommendation of the Microbial Infection and Immunity Microbiology Center for Microbial Interface Biology, The Ohio State University. This additional incubation time is believed to allow for further maturation and improved adhesion of the biofilm to the plate. This

increased adhesion makes the biofilm more durable to withstand the CV assay, particularly the washing steps.

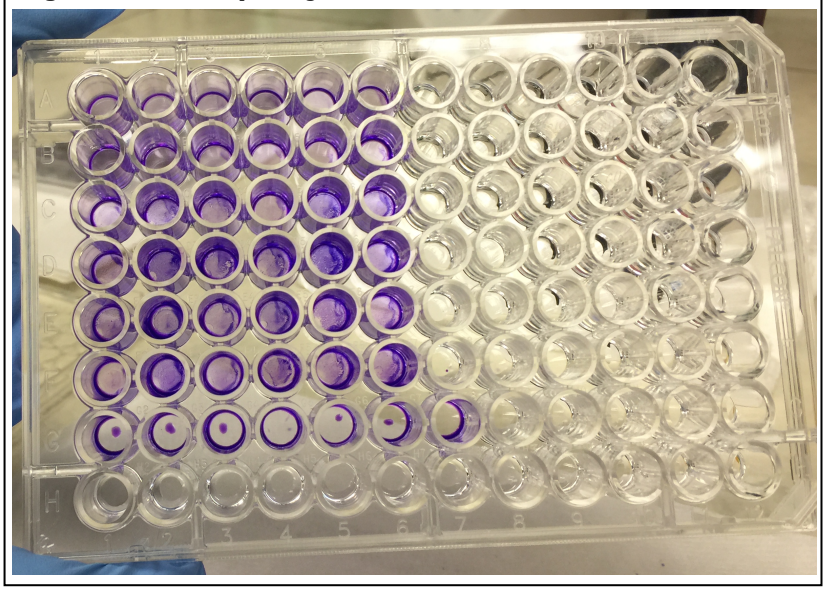
Aspiration

Originally, 180 uL was the suggested cell culture quantity to be aspirated from the wells. However, our observations are that removing this quantity leads to a disturbance of the *S. aureus* growth at the bottom of the well. One possible cause of this disturbance could be that there is an evaporation of media during incubation that leads 180 uL to be an overestimate of removable free cell culture. To avoid the removal or disturbance of intact biofilm, the protocol was modified to aspirate 150 uL before moving onto the wash step.

Washing

The wash steps, which occur twice in the protocol, have the potential to introduce more variability into the results than any other step. Ideally, washing should remove all non-adherent cells while still preserving the integrity of any biofilm.[12] During adaption of the CV assay, two components of the wash required significant modification: the washing technique and the number of washes. In the original protocol, the washing technique began after wells were aspirated. First, plates were inserted into a beaker of distilled water at a 90-degree angle. The plate was then vigorously passed back and forth five times. Immediately

Figure 1: Post-wash pooling



after, it was blotted on paper towels 2-5 times. This technique was difficult to perform efficiently as the plates remained wet inside and out and there was potential for splash of infectious material. It was also difficult to eliminate inconsistently washed wells and pooling of excess CV and water within wells. This was particularly noticeable in the wash after the crystal violet stain; as seen in Figure 1, many wells contained pools of purple tinted water around the perimeter of the bottom of each well even after the final wash step. This leftover material that was not properly removed by the wash, artificially elevated the CV absorbency reading of each well beyond the limit of the plate reader, which did not allow for differentiation between high, moderate, and non-biofilm producers. Inserting the plate into the bucket at a 45-degree angle was a modification made to fix these inconsistencies, but the data was still immeasurable. To further modify the assay, the bucket technique was completely eliminated. Instead, after 150 uL is aspirated from the wells, the plates are forcefully 'blotted' onto paper towels 3 times to remove remaining liquid while preserving the biofilm on the bottom of the wells. Next, 200 uL of double distilled water is pipetted into each well. Immediately after, 180 uL of the water is then pipetted out of the wells. The plates are forcefully 'blotted' again onto paper towels 3 times to remove remaining liquid. This wash is repeated once more. Figure 2, compares a plate created using the bucket technique (top) vs. a plate created using the new blotting technique (bottom). This new washing technique eliminated leftover material such as excess CV, allowing for differentiation between high,

Figure 2: Bucket vs. Blot Washing Technique

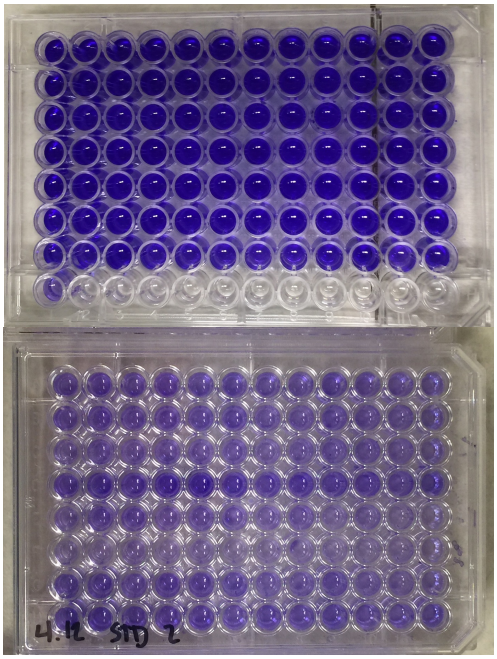


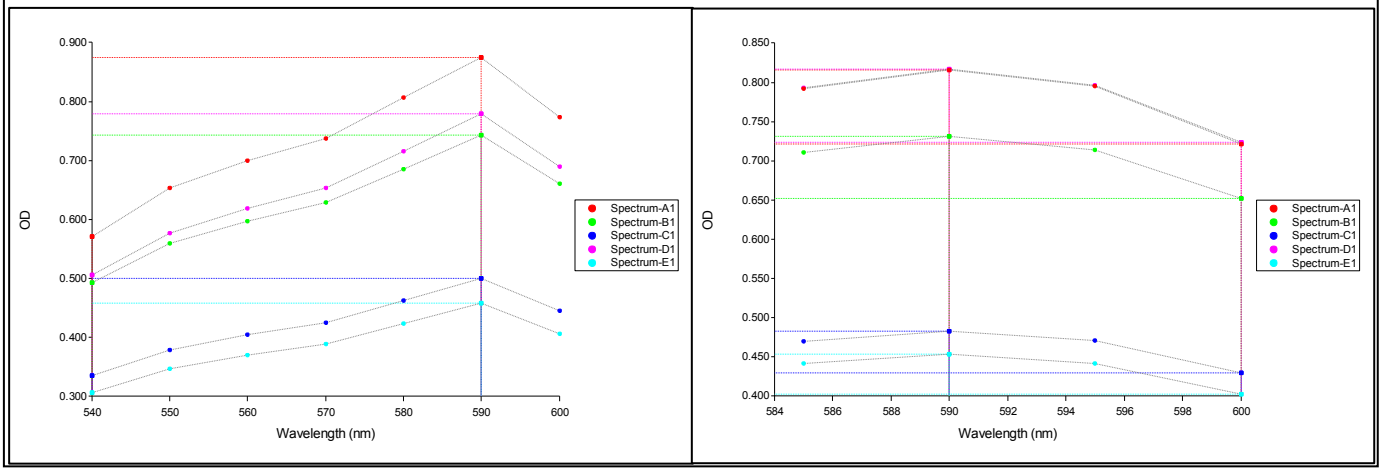
Table 3: Control Strains	
Strain	**Average Absorbance @ 590nm**
NE1241	0.68
WT JE2	0.64
NE1193	0.47
NE95	0.79

moderate, and non-biofilm producers. With this new technique averages for each control strain have been identified after 4 replications of 18-wells/control strain/plate and are seen in Table 3.

Measurement of Results

The original CV assay required the optical density of each well containing solubilized Crystal Violet stained cells be read with a microplate reader at 540 nm. However, a spectrum analysis of 4 plate replicates between 540 and 600nm on an increment of 10nm suggested that the max OD was recorded at 590nm. This was confirmed with an additional spectrum analysis between 580 and 600nm on an increment of 5nm. The magnitude of absorbance is important because we are trying to detect small amounts of material. For this reason it is critical to measure at the most sensitive wavelength, or the wavelength of maximum absorbance, which for this assay is 590nm. Figure 3 shows an example of the two spectrum analyses used to determine an optimal absorbance of 590nm for each plate.

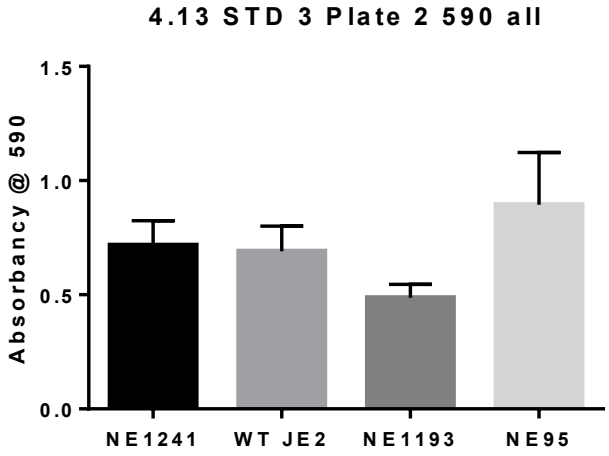
Figure 3: Spectrum analysis 540-600nm and 580-600n



Control Strains

Although NE1241 (nucA-) was intended to perform as a high producing strain, in all trials it's absorbance readings were not statistically greater than WT JE2 ($P < 0.05$). Fortunately, NE95 (agr-) consistently preformed as a high producer (Graph 1). It is important that a high, moderate, and non-biofilm producing strain were identified and verified because the CV assay requires these controls to be plated and compared with clinical isolates to allow for clinical isolate characterization. NE95 was verified as a high biofilm producer, WT JE2 as a moderate biofilm producer, and NE1193 as a non-biofilm producer.

Graph 1: Control isolate absorbance @ 590nm



CONCLUSIONS

This study identified the most critical points of the CV assay that required optimization for future clinical applications. Changing the quantity of overnight added to TSB as well as the glassware involved in the overday culture growth resolved the issues of subsampling and slow growth. Increasing overnight incubation time allowed for more mature and durable biofilm growth within the 96-well plates. Decreasing aspiration quantities better preserved any biofilm growth on the bottom of the well. Revising the washing technique to involve blotting and pipetting rather than a bucket more thoroughly removed excess cells, water, and CV stain. Preforming spectrum scans using a microplate reader confirmed a revision in the optimal absorbance wavelength in which plates should be read. All of these changes have lead to the

optimization of the CV assay. In the future, this standardized Crystal Violet assay for quantifying biofilm mass can be implemented to screen the biofilm production capabilities of *S. aureus* isolates obtained from a veterinary teaching hospital during routine surveillance over a span of seven years. Through this method, using the defined control strains, the biofilm production of a clinical isolate can be classified as high, moderate, or non-producing. This data can be incorporate with previous epidemiological information associated with each isolate. The information obtained could help to better understand the potential role of biofilm production in the ecology and epidemiology of *S. aureus* in veterinary hospitals. This information can then be used to revise and enhance cleaning and disinfecting protocols to better ensure the safety of patients and hospital staff.

ACKNOWLEDGEMENTS

We wish to thank Dr. Daniel Wozniak and his graduate student Mohini Bhattacharya, from the Center for Microbial Interface Biology, The Ohio State University, for providing the original CV assay protocol and the control strains for the standardization of the CV assay. We are grateful for the financial support provided for the development of this project by The Ohio State University Undergraduate Research Office and The Ohio State University College of Food, Agricultural, and Environmental Sciences.

BIBLIOGRAPHY

1. Klein E. Smith D. Laxminarayan R. Hospitalizations and deaths cause by methicillant resistant Staphylococcus aureus, United States, 1999–2005. Emerg Infect Dis. 2007;13:1840–1846.
2. Pantosti A. Methicillin-resistant Staphylococcus aureus associated with animals and its relevance to human health. Front Microbiol. 2012;3:127.
3. Van Balen, J., Kelley, C., Nava-Hoet, R. C., Bateman, S., Hillier, A., Dyce, J., & Hoet, A. E. (2013). Presence, distribution, and molecular epidemiology of methicillin-resistant Staphylococcus aureus in a small animal teaching hospital: a year-long active surveillance targeting dogs and their environment. *Vector-Borne and Zoonotic Diseases*, *13*(5), 299-311.
4. Hoet, Armando E., et al. "Epidemiological profiling of methicillin-resistant Staphylococcus aureus-positive dogs arriving at a veterinary teaching hospital." *Vector-Borne and Zoonotic Diseases* 13.6 (2013): 385-393.
5. Singh, A., Walker, M., Rousseau, J., & Weese, J. S. (2013). Characterization of the biofilm forming ability of Staphylococcus pseudintermedius from dogs. *BMC veterinary research*, *9*(1), 93.
6. Turk, R., Singh, A., Rousseau, J., & Weese, J. S. (2013). In vitro evaluation of DispersinB on methicillin-resistant Staphylococcus pseudintermediu biofilm. *Veterinary microbiology*, *166*(3), 576-579.
7. Clutterbuck, A. L., Woods, E. J., Knottenbelt, D. C., Clegg, P. D., Cochrane, C. A., & Percival, S. L. (2007). Biofilms and their relevance to veterinary medicine. *Veterinary microbiology*, *121*(1), 1-17.
8. Smith, K., & Hunter, I. S. (2008). Efficacy of common hospital biocides with biofilms of multi-drug resistant clinical isolates. *Journal of medical microbiology*, *57*(8), 966-973.
9. Croes, Sander, et al. "Staphylococcus aureus biofilm formation at the physiologic glucose concentration depends on the S. aureus lineage." *BMC microbiology* 9.1 (2009): 1.
10. Otto, Michael. "Staphylococcal biofilms." *Bacterial biofilms*. Springer Berlin Heidelberg, 2008. 207-228.
11. Donlan, R. M., & Costerton, J. W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical microbiology reviews*, *15*(2), 167-193.
12. Stepanović, Srdjan, et al. "Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci." *Apmis* 115.8 (2007): 891-899.
13. Burton, E., et al. "A microplate spectrofluorometric assay for bacterial biofilms." *Journal of industrial microbiology & biotechnology* 34.1 (2007): 1-4.
14. Boles, Blaise R., and Alexander R. Horswill. "Agr-mediated dispersal of Staphylococcus aureus biofilms." *PLoS Pathog* 4.4 (2008): e1000052.
15. Moormeier, Derek E., et al. "Temporal and stochastic control of Staphylococcus aureus biofilm development." *MBio* 5.5 (2014): e01341-14.
16. Kiedrowski, Megan R., et al. "Nuclease modulates biofilm formation in community-associated methicillin-resistant Staphylococcus aureus." *PLoS One* 6.11 (2011): e26714.

17. Cheung, Ambrose L., et al. "Regulation of exoprotein expression in Staphylococcus aureus by a locus (sar) distinct from agr." *Proceedings of the National Academy of Sciences* 89.14 (1992): 6462-6466.
18. Beenken, Karen E., Jon S. Blevins, and Mark S. Smeltzer. "Mutation of sarA in Staphylococcus aureus limits biofilm formation." *Infection and Immunity* 71.7 (2003): 4206-4211.